Vol. 41, No. 1, January 1997 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 169-177

EXPRESSION AND RECONSTITUTION OF CALCINEURIN A AND B SUBUNITS

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Received November 4, 1996
Received after revision November 15, 1996

SUMMARY. Calcineurin consists of two subunits, a catalytic A subunit of 60 kDa and a regulatory B subunit of 19 kDa. Both the A and B subunits of rat brain calcineurin were expressed in *E. coli*. The B subunit was readily overexpressed in the pET-21a vector with yields of >70 mg of purified B subunit per l culture, representing >17% of the soluble *E. coli* protein. About 8 mg of purified A subunit was obtained. The enzyme activities of the A-subunit and the reconstituted AB complex were found to be comparable to that of the bovine brain enzyme. The reconstitution of the AB complex was studied and shown to be rapid.

Key words: protein phosphatase, expression, calcineurin, calmodulin.

INTRODUCTION.

Calcineurin is a calcium/calmodulin-dependent protein phosphatase (1; for reviews, see 2-6). The enzyme consists of two subunits, the catalytic A subunit of 60 kDa (CaN A), and a regulatory B subunit (CaN B) of 19 kDa. Inhibitor-1, the inhibitor protein of protein phosphatase-1, is a preferred substrate, as is the neural protein, DARPP-32 (4,5). Although calcineurin is present in all mammalian cells, it is most highly abundant in brain tissue (2,3,6). While the biological functions of calcineurin in the brain have not been completely elucidated, it is most highly concentrated in the hippocampus, and may play a role in regulating neurotransmitter action (6). An important role of calcineurin has been revealed by the finding that it is the target of the immunosuppressant drugs, FK506 and cyclosporin A, both of these being inhibitory as their complexes with their respective immunophilin proteins (7). Multiple cDNAs have been isolated for the mammalian CaN A subunit (8-13) There are three mammalian genes for the A subunit, giving rise to α, β and γ isoforms (2). The α gene gives rise to two transcripts, differing by a 10-amino acid insert at the C-terminus (11). Two isoforms of the CaN B subunit have been cloned (14,15). In this study we report the high level expression of the CaN B subunit and the full length CaN A subunit in *E. coli*, their isolation and their reconstitution into a fully active heterodimer.

1039-9712/97/010169-09$05.00/0

MATERIALS AND METHODS.

Materials. The cDNAs for the αδ isoform of CaN A (16,17) and CaN B (18) were those isolated from rat brain cDNA libraries, and were generous gifts of Drs B. Perrino and T.R. Soderling, Vollum Institute, Portland, Oregon. The protein encoded by the rat brain CaN B cDNA (18) has an amino acid sequence identical that of human CaN B (19). Calmodulin was isolated from bovine brain (20) and calmodulin-Sepharose was prepared by coupling to CNBr-activated Sepharose (Pharmacia-LKB Biotech). Polyclonal antibodies against the A and B subunits of bovine brain calcineurin were raised in mice using methods previously described (21). Phenyl-Sepharose, Sephacryl S-200 and CNBr-activated Sepharose were obtained from Pharmacia-LKB Biotech. Restriction enzymes were obtained from New England Biolabs. Protein sequence analyses were performed by the Protein Chemistry Core, ICBR, University of Florida, Gainesville.

Construction of vectors and expression. The coding sequences of both the CaN A and CaN B subunits were isolated by PCR amplification. For CaN A, NdeI and HindIII restriction sites were engineered at the 5' and 3' ends, respectively, using the primers 5'-CGCGCATATGTCCGAGCC CAAGGCG-3' and 5'-CGCGAAGCTTTCACTGAATATTGCTGC-3'. The PCR fragment was subcloned into the pET-21a expression vector (Novagen) using the NdeI and HindIII restriction sites. Expression of CaN A was performed in *E. coli* HMS174(DE3) host cells grown at 37°C in Terrific media (1.2% tryptone, 2.4% yeast extract, 0.4 % glycerol, 0.017 M KH_2PO_4, and 0.072 M K_2HPO_4, containing 50 μg/ml ampicillin) in 1 liter cultures. Cells were grown at 37°C until the optical density at 600nm reached a value of 0.7. Expression was then induced by addition of 0.5 mM isopropyl β-thiogalactoside (IPTG) followed by growth for an additional 4 hr at 37°C. For CaN B, NdeI and BamHI sites were engineered at the 5' and 3' ends of the coding sequence using the primers 5'-CCGCCATATGGGAAATGAGGCGAGTT-3' and 5'-CGCGGGATCCTCACAC ATCTACCACCA-3', respectively. The PCR fragment was then inserted into the pET21a vector using the NdeI and BamHI sites. BL21(DE3)plysS cells were used for expression of the CaN B subunit. For CaN B, expression was found to be constitutive when the cells were grown in Terrific media plus 0.6% glycerol. The sequences of the CaN A and CaN B inserts were confirmed by dideoxynucleotide sequencing.

Purification of recombinant CaN A. The cells from 1 liter cultures were harvested by centrifugation at 4000 x g at 4°C for 20 minutes and resuspended in 1/10th volume of buffer A (100 ml) which contained 50 mM TrisHCl, 1 mM EDTA, 1 mM DTT (dithiothreitol), pH 7.4. The cells were disrupted by passage through a French press. The lysate was then centrifuged at 30,000 x g for 1 hr at 4°C. The supernatant was precipitated by addition of ammonium sulfate to 50% saturation. After centrifugation, the pellets were resuspended in buffer A and dialyzed against three changes of buffer A. Calcium chloride was added to the dialysate to a final concentration of 3 mM. This was then mixed with CaM-Sepharose 4B (8 ml) which was equilibrated with 20 mM TrisHCl, 1 mM DTT, 0.5 mM $CaCl_2$, pH 7.4. The suspension was gently mixed by rotation for several hours or overnight at 4°C. The gel was poured into a column, washed with the equilibrating buffer, and eluted with 20 mM TrisHCl, 1 mM DTT, 1 mM EGTA, pH 7.4. The eluted fractions were concentrated by centrifugation through Centriplus 30 membranes (Amicon). The eluate was loaded into a Sephacryl-200 column (1 x 60 cm), equilibrated with Buffer A plus 0.2M NaCl. The active fractions were collected and concentrated by centrifugation through Centriplus 30 membranes. The purified material was brought to 50% glycerol and stored at -20 C. Recovery of CaN A protein was generally from 7-10 mg per preparation.

Purification of recombinant CaN B. Lysates of cells from 1 liter cultures were prepared as for CaN A. The crude lysate was heated at 100°C for 30 min and centrifuged at 15,000 x g for 15 min. The supernatant was loaded onto a phenyl-Sepharose column (2 x 20 cm) equilibrated with 20 mM TrisHCl, pH 7.4, 1 mM DTT, 0.5 mM Ca^{2+}. The column was washed with same buffer and then eluted with 20 mM Tris pH 7.4 , 1 mM DTT, 1 mM EGTA. Recovery of protein was from 70 to 80 mg of pure CaN B.

Phosphatase assay. The activity of the CaN A subunit and the reconstituted CaN AB heterodimer was assayed using p-nitrophenyl phosphate (PNPP) as the substrate (22). The assay contained 50 mM TrisHCl, pH 7.4, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.5 mM $MnCl_2$, 0.2 mg/ml bovine serum albumin, 0.3 μM CaM, 20 mM PNPP. The enzyme was assayed for 20 min. at 30° C in a final volume of 200 μl. Reactions were terminated by the addition of 1.8 ml 0.5 M Na_2CO_3, 20 mM

EDTA and the absorbance at 410 nm was measured using a control lacking enzyme. One unit of activity was defined as that catalyzing the hydrolysis of 1 nmole of p-nitrophenyl phosphate/min. *Protein determinations.* Protein determinations were performed by the Bradford method (23).

RESULTS.

Expression and isolation of the CaN B subunit. The cDNA of the rat brain CaN B was inserted into the pET-21a vector. The expression after induction with IPTG was found to relatively poor; however, during examination of the effects of various growth conditions on expression, it was observed that the CaN B subunit was strongly expressed in growth media containing 0.6% glycerol, even in the absence of added IPTG. The reasons for the resistance of the pET21a/CaN B construct to IPTG induction, and the response to glycerol are unknown. This phenomenon was not observed with the CaN A subunit in the same vector. Nevertheless, the behavior of the system was highly reproducible, and allowed for the isolation of relatively large amounts of CaN B. The purification protocol used was a two-step procedure. The *E. coli* lysate was heat-treated at 100°C, after which the 16 kDa CaN polypeptide appeared as the major component on SDS-PAGE and could be purified to homogeneity by phenyl-Sepharose chromatography (24). The SDS-PAGE of the material after heat-treatment and phenyl-Sepharose chromatography are shown in Fig. 1. The yield of CaN B was usually over 70 mg protein/liter culture. The level of expression was estimated to be ca. 17% of the soluble *E. coli* protein assuming a 100% recovery (Table I). The identity of the protein as the CaN B subunit was established by its functional ability to activate the CaN A subunit and by immunoblotting with an antibody against bovine calcineurin B subunit (not shown). In view of the unusual behavior of the expression system, N-terminal amino acid sequencing of the recombinant CaN B was performed. The N-terminal sequence obtained was GNEASYPLEM, confirming the identity of the protein as well as the fact that initiation of transcription was at the appropriate ATG start codon (19).

Expression of the A-subunit. The coding sequence of the CaN A αδ isoform was inserted into the pET-21a vector. Expression of CaN A was followed by immunoblotting and by activity assay. After induction with IPTG, immunoreactive protein was distributed in both the insoluble and soluble fractions of the lysates. However, the amounts of protein present in the soluble fraction were sufficient to allow for isolation of workable amounts of protein. The soluble CaN A protein was found to be active in the presence of Mn^{2+} and was assayed by its activity on p-nitrophenyl phosphate (Materials and Methods). Purification of CaN A was performed by a simple three step procedure based on the method of Klee *et al.* (25), involving ammonium sulfate precipitation, affinity chromatography on calmodulin-Sepharose, and finally gel filtration on Sephacryl S-200. The SDS-PAGE of the material at different stages of purification is shown in Fig. 2. After affinity chromatography on calmodulin-Sepharose, the major polypeptide is a 60 kDa protein. Minor impurities were largely removed after Sephacryl S-200 chromatography (Fig. 2). Identification of the 60 kDa band as CaN A subunit was established by Western blotting (Fig. 2, lane 4).

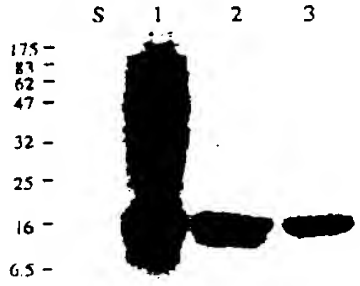

Fig. 1. Purification of CaN B expressed in *E. coli*.
Samples of the preparations were subjected to SDS-PAGE. Lane S, protein standards (New England Biolabs); lane 1, crude lysate, lane 2, after heat-treatment and lane 3, after phenyl-Sepharose chromatography.

Table I. Purification of Recombinant Calcineurin B-Subunit.

	Vol. (ml)	Protein (mg)	Recovery (%)
Crude lysate	102	449	100
Heat-treated extract	94	126	28
Phenyl-Sepharose	168	76	17

The overall recoveries of protein are shown in Table II. In the preparation shown, 7.7 mg protein was recovered from the lysate originating from 1 liter of cell culture. The CaN A subunit was inactive but could be activated by Mn^{2+}, as has been previously reported for the bovine brain CaN A subunit (1,2). The overall recovery of activity was 27%, with a 7-fold purification from the crude lysate, representing an expression level of 14% of the soluble *E. coli* protein.

Characterization of the A-subunit. As noted above, the A subunit exhibited activity toward p-nitrophenyl phosphate, provided Mn^{2+} was present. The specific activity of the CaN A preparations ranged from 80-100 units/mg. The response of the CaN A subunit to various additions is shown in Fig. 3. The addition of calcium or calmodulin alone had only a slight effect on the activity, but in the presence of both there was a greater than 2-fold increase in activity. When the B subunit was present, activity was roughly doubled. Further addition of calcium or calmodulin alone had only slight effects, as with free CaN A. Addition of both led to a further 2-fold stimulation. Maximal stimulation to the full specific activity occurred in the presence of both calcium, calmodulin and the B subunit. Overall, the behavior of the enzymatic activity of the isolated A subunit, and the reconstituted holoenzyme, were highly consistent with that observed

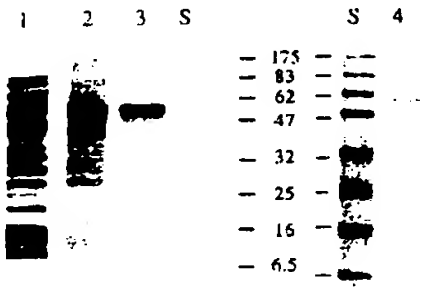

Fig. 2. Purification of CaN A subunit expressed in *E. coli*.
The CaN A subunit was expressed using the pET21a vector and purified by ammonium sulfate precipitation, affinity chromatography on calmodulin-Sepharose and gel filtration on Sephacryl S 200 (Materials and Methods). The diagram shows the SDS-PAGE stained for protein of the material obtained at different stages of the preparation. Lanes 1, 2, and 3, are the crude lysate, material after CaM-Sepharose and Sephacryl S-200 chromatography, respectively. Lane 4 (right hand panel) is a Western blot of the material obtained after Sephacryl S-200 chromatography using an antibody against bovine brain calcineurin A-subunit. Protein standards (New England Biolabs) are shown by the lines and are given in kDa.

Table II. Purification of Recombinant Calcineurin A-Subunit.

	Vol. (ml)	Protein (mg)	Activity (units)	Sp. Act. (units/mg)	Recovery (%)	Purif. fold
Crude extract	101	202	2,500	14	100	1.0
50% Amm. Sulf.	26	117	2,244	19	68	1.4
CaM-Sepharose	27	10	826	80	30	6.0
Sephacryl-200	30	7.7	733	95	27	7.0

for the subunits expressed in Sf9 cells by Perrino *et al.* (16,18). The reconstitution of the recombinant CaN A and CaN B subunits apparently took place rapidly, as no time lag was detected when the CaN A subunit was pre-incubated at 0°C for 0, 1 and 3 hr with increasing concentrations of the CaN B subunit (Fig. 4). In this experiment, the starting concentration of CaN A was 0.5 μM, and it is seen that maximal activity was observed at ca. 0.5μM CaN B, indicating a 1:1 stoichiometry.

In order to ascertain that a physical complex was formed, the behavior of the CaN A subunit and the complex were examined by gel filtration chromatography on Sephacryl S-200. The CaN A subunit, and a mixture of the CaN A subunit with an excess of the CaN B subunit were chromatographed. The peak fractions of the chromatography of the A + B mixture were western blotted. The results (Fig. 5 insert) show the co-migration of CaN B subunit with the CaN A subunit, confirming the physical formation of a complex. In addition, when a mixture of CaN A with excess CaN B was chromatographed on calmodulin-Sepharose in the presence of calcium,

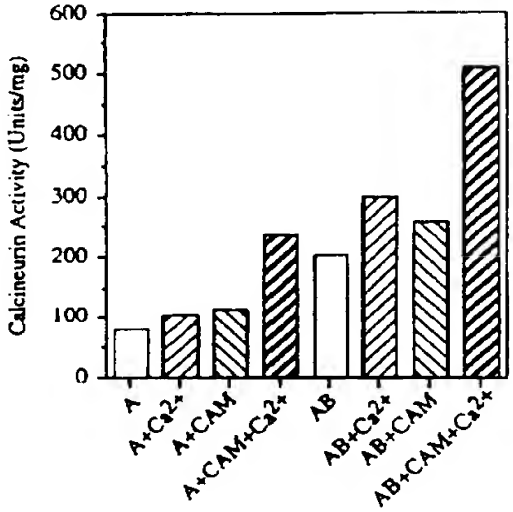

Fig. 3. Effects of CaN B, Ca^{2+} and calmodulin on the activity of recombinant CaN A.
CaN A activity was assayed by the hydrolysis of p-nitrophenyl phosphate as described in Materials and Methods. Data are shown as units/mg CaN A protein. CaN B, calmodulin (CAM), and Ca^{2+}, where added, were at concentrations of 0.5 μM, 0.3 μM and 0.2 mM, respectively. All the assays performed were carried out in the presence of 0.5 mM Mn^{2+}.

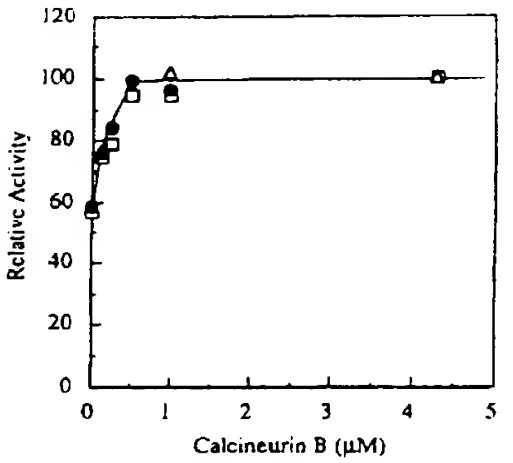

Fig. 4. Effects of preincubation of CaN A and CaN B on enzyme activity.
CaN A (0.5μM) was preincubated at 0°C for 0 hr (solid circles), 1 hr (open squares) and 3 hr (open triangles) with the indicated concentrations of CaN B before assay in the presence of calmodulin and calcium. Data are expressed as relative activities.

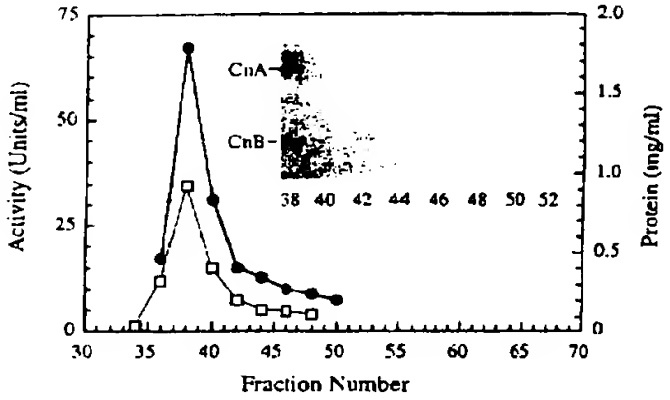

Fig. 5. Sephacryl S-200 chromatography of reconstituted calcineurin. CaN A (1 mg) and CaN B (1 mg) were mixed in a total volume of 0.5 ml and chromatographed on a Sephacryl S-200 column (1 x 60 cm) equilibrated with 50 mM imidazole chloride, pH 7.4, 1 mM DTT, 1 mM $CaCl_2$ and 50 mM NaCl. Fractions of 0.5 ml were collected and assayed for enzyme activity (solid circles) and protein (open squares). The inset shows the western blotting of the fractions with a mixture of antibodies to CaN A and CaN B.

it was observed that the excess CaN B was not bound to the column, and a complex of CaN A and CaN B could then be eluted with EDTA (not shown). This procedure was used for preparative scale reconstitution of the CaN AB heterodimer. The reconstituted enzyme exhibited specific activities of 250-350 units/mg heterodimer protein.

DISCUSSION.

We have developed a facile system for the expression of the rat brain αδ isoform of the CaN A subunit, and of the rat brain CaN B subunit. The levels of expression were sufficiently high to make this system useful for production of the enzyme in amounts sufficient for isolation and study. Rat CaN B has been expressed in Sf9 cells using a baculovirus expression vector (18), and human CaN B in *E. coli* (24,26). Both methods yielded useful amounts of protein, although the yields were not reported for the expression in insect cells. Expression of human CaN B in *E. coli* using the pET-9d vector was reported to provide 5-10 mg protein/liter of cell culture, about 1/10th of that which we obtained.

The rat CaN A αδ isoform we used has been expressed in baculovirus (16) and as a fusion protein in *E. coli* with nine residues of the T7 gene 10 protein at the N-terminus (27). The human CaN A isoform containing a polyproline region in the N-terminus (8) has been expressed as an insoluble glutathione-S-transferase (GST) fusion protein in *E. coli* by Rokosz *et al.* (26). The human CaN A isoform was poorly expressed in *E. coli* despite the use of a number of different vectors, and expression was only obtained as the GST-fusion protein. In addition, there were

significant problems with proteolytic degradation. Reconstitution of the enzyme required cleavage of the GST fusion protein, followed by renaturation from urea solutions of the protein. However, the renaturation of the CaN A subunit from the urea solutions was dependent on the presence of the B subunit and calmodulin, so that this procedure did not allow isolation of the free A-subunit (26). Furthermore, the reconstituted enzyme could not be isolated in a pure form, and the preparation was estimated to have a purity of 38%. Successful expression and isolation of the CaN A αδ isoform has achieved in baculovirus by Perrino *et al.* (16), although the yields were not reported. The functional behavior of the bacterially expressed CaN A-subunit reported in our studies, as tested by the hydrolysis of PNPP, are very similar to those reported by Perrino *et al.* (16) for the protein expressed in Sf9 cells. The specific activity of the reconstituted enzyme is also comparable to values reported for the reconstituted enzyme produced from Sf9 cells, and to literature values for the bovine brain enzyme (16,25). The rat CaN Aα fusion protein expressed in *E. coli* (27) was distributed in both particulate and soluble fractions, similar to the results obtained in this study, but yields of protein were not reported.

The reconstitution of the recombinant A subunit with the B subunit was found to proceed rapidly and with a maximal activation of activity at a 1:1 stoichiometry, an ideal behavior which has not been previously observed. Reconstitution of the bovine brain A subunit isolated after urea dissociation was found to require up to 5 hours at 30°C (28), while the Sf9 expressed enzyme required 3 hours of preincubation (16). In addition, maximal stimulation of the bovine brain A subunit occurred at a molar ratio of subunit B/subunit A of 3 (28). A possible explanation for the difference in our experiments is that the CaN A which we isolated is in a properly folded native conformation, allowing for rapid reconstitution of the complex. It should be noted that the CaN A subunit was purified by affinity chromatography on CaM-Sepharose. This could lead to a positive selection for those molecules which are in a fully native conformation.

In summary, we have developed an expression system in *E. coli* that permits high level expression of the CaN A and CaN B subunits, and have shown that the enzyme can be readily reconstituted in an active form that behaves like the native enzyme. This system should facilitate structure-function studies of the enzyme, given also the fact that the crystal structure of calcineurin has now been solved (29,30).

Acknowledgments. This work was supported by NIH Grant DK18512.

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